N-succinylamino acid racemases: enzymatic properties and biotechnological applications

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Abbreviations: *N*-succinylamino acid racemase (NSAR); o-succinylbenzoate synthase (OSBS); *N*-succinylamino acid racemase/o-succinylbenzoate synthase subfamily (NSAR/OSBS); *N*-acetyl-amino or *N*-acyl-amino acid racemase (NAAR); *N*-substituted-amino acid (NxA); *N*-substituted-amino acid racemase (NxAR); Kinetic resolution (KR); Dynamic kinetic resolution (DKR); *N*-acetyl- (NA); *N*-acetyl-amino acid (NAA); *N*-succinyl- (NS); *N*-succinyl-amino acid (NSA); *N*-carbamoyl- (NC); *N*-carbamoyl-amino acid (NCAA); *N*-chloroacetyl- (NCh); *N*-chloroacetyl-amino acid (NChAA); *N*-formyl- (NF); *N*-formyl-amino acid (NFAA); *N*-butyril (NBt); *N*-butyril amino acid (NBtA); *N*-propionyl (NPr); *N*-propionyl amino acid (NPrA); *N*-benzoyl (NBzA); *N*-benzoyl amino acid (NBzA); (1R,6R)-2-succinyl-6-hydroxy-2,4-cyclohexadiene-1-carboxylate (SHCHC); *Amycolatopsis* sp. TS-1-60 NSAR (AmyNSAR); *Geobacillus kaustophilus* NSAR (GkaNSAR); *Geobacillus stearothermophilus* CECT 49 NSAR (GstNSAR); *Streptomyces atratus* Y-53 NSAR (SatNSAR); *Sebekia benihana* NSAR (SebeNSAR); *Chloroflexus aurantiacus* NSAR (CauNSAR); *Thermus thermophilus* NSAR (TteNSAR); *Deinococcus radiodurans* NSAR (DraNSAR); *Exiguobacterium* sp. AT1b OSBS (ExiOSBS); *Alicyclobacillus*

acidocaldarius OSBS (AacOSBS); *Enterococcus faecalis* V583 NSAR (EfaNSAR); *Listeria innocua* Clip11262 NSAR (LinNSAR); *Lysinibacillus varians* GY32 NSAR (LvaNSAR); *Roseiflexus castenholzii* HLO8 NSAR (RcaNSAR); *Amycolatopsis mediterranei* S699 NSAR (AmedNSAR).

ABSTRACT

The *N*-succinylamino acid racemase/o-succinylbenzoate synthase (NSAR/OSBS) subfamily from the enolase superfamily contains different enzymes showing promiscuous *N*-substituted-amino acid racemase (NxAR) activity. These enzymes were originally named as *N*-acylamino acid racemases because of their industrial application. Nonetheless, they are pivotal in several enzymatic cascades due to their versatility to catalyze a wide substrate spectrum, allowing the production of optically pure D- or L-amino acids from cheap precursors. These compounds are of paramount economic interest, since they are used as food additives, in the pharmaceutical and cosmetics industries and/or as chiral synthons in organic synthesis. Despite its economic importance, the discovery of new *N*-succinylamino acid racemases has become elusive, since classical sequence-based annotation methods proved ineffective in their identification, due to a high sequence similarity among the members of the enolase superfamily. During the last decade, deeper investigations into different members of the NSAR/OSBS subfamily have shed light on the classification and identification of NSAR enzymes with NxAR activity of biotechnological potential. This review aims to gather the dispersed information on NSAR/OSBS members showing NxAR activity over recent decades, focusing on their biotechnological applications and providing practical advice to identify new enzymes.

Introduction

As for other enzymatic racemization methodologies involved in dynamic kinetic resolution (DKR) processes, the driving force boosting *N*-succinylamino acid racemase (NSAR)[1] research is most likely the existence of a previous enzymatic kinetic resolution (KR) method for the production of optically pure amino acids. This KR process was

[1] We will use different acronyms (NSAR, NAAR) through the manuscript to refer to enzymes belonging to the N-succinylamino acid racemase/o-succinylbenzoate synthase (NSAR/OSBS) subfamily.

already known since middle of the twentieth century [1], and takes advantage of the stereospecificity of acylases (or aminoacylases, E.C. 3.5.1.14) to produce optically pure amino acids from racemic mixtures of *N*-acetylamino acids (NAAs) [2, and references therein]. Evonik (Degussa) industries already manufactured L-methionine at industrial level in the 70`s using the "Acylase process" [3]. Furthermore, patents on the chemical racemization of NAAs by different companies clearly illustrate the interest to develop a chemo-enzymatic DKR before the eighties (e.g., Ajinomoto or Evonik/Degussa [4,5]). Takeda Pharmaceutical Co Ltd discovered the *N*-acylamino acid racemase (NAAR) activity in different microorganisms by the end of the 80's, demonstrating a potential NAAR/acylase enzymatic tandem, also providing a new DKR approach for optically pure D- and L-amino acids [6-8]. The industrial relevance of these enzymes is clear by the extensive number of patents issued [6-43] (Table 1).

To the best of our knowledge, the first paper dealing with an NSAR enzyme was not published until the 90s. Tokuyama's group described NAAR activity in some actinomycetes strains belonging to the genus *Streptomyces*, *Actinomadura*, *Actinomyces*, *Jensenia*, *Amycolatopsis* and *Sebekia*. However, they did not find this activity in other bacteria, yeasts or fungi, from more than 44000 strains tested [44,45]. From the organisms described in that work, enzymes belonging to *Streptomyces atratus* Y-53 (SatNSAR) [46], *Sebekia benihana* (SebeNSAR) [22-24] and *Amycolatopsis* [47-49] were characterized and used (Table 1). Whereas the principal interest of these enzymes was the racemization of NAAs, their ability to racemize different *N*-substituted-amino acids (NxAs) was also soon reported, showing them to be active towards *N*-acetyl (NAAs), *N*-carbamoyl (NCAs), *N*-chloroacetyl (NChAs), *N*-butyril (NBtAs), *N*-propionil (NPrAs), *N*-benzoyl (NBzAs) or *N*-formyl-amino acids (NFAs), among others [46,47]. Despite the potential DKR application of these enzymes, only a discrete number of NAAR enzymes were identified and/or characterized in the following years, mainly from *Amycolatopsis* and *Deinococcus* genus, mostly boosted by their industrial interest [25, 50-53, see also Table 1] or for structural characterization [54,55]. Additional actinomycetes strains were shown to possess NAAR activity, although no further information has been reported on the enzymes from these microorganisms [56]. NSAR was already shown to be a member of the functional diverse enolase superfamily before the beginning of the new millennium [51]. Several works at the beginning of the 21st century focused on deciphering the natural function of

NSARs and some evolutionary aspects [51,57,58, 69-76] (see below), allowing to localize new NSARs in *Bacillus*/*Geobacillus* genera. Some of the NSARs belonging to this genera have been highly characterized and/or applied to the production of optically pure amino acids [27-38,59,61-68]. A different *N*-succinyl arginine/lysine racemase from *Bacillus cereus* ATCC 14579, highly specific for NS-Arg and NS-Lys has been reported, although it does not belong to the NSAR group reviewed in this paper [59]. The characterization of an NSAR from *Chloroflexus aurantiacus* (CauNSAR) [26] and the determination of the crystal structure of *Thermus thermophilus* NSAR (TteNSAR) [60], further expand the organisms in which NSAR activity has been found. Finally, *Exiguobacterium*, *Lysinibacillus*, *Roseiflexus*, *Enterococcus* or *Listeria* genera have also been reported to present NSAR activity, although they have been studied in less detail from the biotechnological point of view [72-74]

Natural function and evolutionary aspects on NSAR enzymes

After one decade using the industrially-biased NAAR nomenclature, Gerlt's group gave an important step towards understanding the natural function of NSAR, suggesting that the "correct" function of *Amycolatopsis* sp. TS-1-60 NAAR (AmyNSAR) was the o-succinylbenzoate synthase (OSBS) reaction in menaquinone biosynthesis [51], due to the sequence similarity to *Bacillus subtilis* OSBS. The NAAR activity was suggested as a secondary and promiscuous activity of OSBS, with no impact on the organism in its metabolic niche (Figure 1). Despite the significance of this work in the comprehension of the promiscuous NSAR activity, the NAAR nomenclature continued to be used for several years in the literature. Another milestone in the understanding of the function of NSAR enzymes was achieved in 2006 [57]. *Geobacillus kaustophilus* was shown to contain a member of the enolase superfamily with NSAR activity (GkaNSAR), homolog to the well-characterized enzymes from *Amycolatopsis*. GkaNSAR was encoded in a new gene cluster, different to the menaquinone biosynthesis operon in which OSBS is included [51] (Figure 2). A similar cluster was identified for *Amycolatopsis* and *Thermus* genera [57] (Figure 2). After two decades of the discovery of NSARs, the authors concluded that GkaNSAR constitutes part of a novel, irreversible pathway for the conversion of D- to L-amino acids (Figure 2, lower panel), different to the previously proposed menaquinone biosynthesis [51]. Since i) this enzyme was functionally promiscuous, also catalyzing efficiently the OSBS reaction, and ii) no other

potential OSBS was encoded in the menaquinone operon or elsewhere in the genome, the authors raised the possibility that the NSAR is a bifunctional enzyme rather than an accidentally promiscuous enzyme, as previously suggested [51]. The nomenclature OSBS/NSAR subfamily was proposed, due to the probable overlapping activity of both enzymes. A seminal and must-read paper by Glasner et al. on the evolution of the OSBS/NAAR subfamily showed that NSARs of known activity were phylogenetically included under the so-called Firmicutes OSBS/NSAR subfamily (Figure 3), together with other enzymes for which NSAR activity could not be detected [58]. This work showed the complex scenario of functional evolution in this subfamily, and helps explaining why only a discrete number of biotechnologically relevant NSAR enzymes had been discovered by that time, since sequence-based approaches also identified OSBS enzymes not necessarily containing NSAR activity (see below).

Patent No.	Description	Pub. year	Organism	Ref
EP0304021A2	Acylamino acid racemase, production and use thereof [1]	1989	*S. atratus*, NSAR-producing microorganisms	[6]
JPH01137973A	Acylamino acid racemase, its production and use[1]	1989	*S. atratus*	[7]
US4981799A	Acylamino acid racemase, production and use thereof[1]	1991	*S. atratus*	[8]
CN1035320A	Acylamino acid racemase and its use[1]	1989	NSAR-producing microorganisms	[9]
JPH06205668A	Bacteria producing acylamino acid racemase[1]	1994	*Amycolatopsis* sp.	[10]
EP0474965A2*	DNA encoding acylamino acid racemase and its use[1]	1992	*S. atratus*, *Amycolatopsis* sp.	[11]
US5525501A*	DNA Fragment encoding acylamino acid racemase[1]	1996	*S. atratus Amycolatopsis* sp.	[12]
CA2038202A1*	DNA and use thereof[1]	1992	*Amycolatopsis* sp.	[13]
JP3066473B2*	DNA and its applications[1]	2000	*Amycolatopsis* sp.	[14]
DE10050123A1	A process for the production of amino acids[2]	2002	*Amycolatopsis orientalis*	[15]
US6656710B2	Process for the production of amino acids using racemase and acylase[2]	2003	*S. atratus*, *Amycolatopsis* sp. and *orientalis*	[16]
DE10050124A1	Using the acetylamino from *Amycolatopsis orientalis* racemization of carbamoylamino[2]…	2002	*Amycolatopsis orientalis*	[17]
EP1074628 A1#	N-Acetylamino acid racemase[2]	2001	*Amycolatopsis orientalis*	[18]
JP2001046088A#	N-acetylamino acid racemase, its coating gene, plasmid, vector and microorganism…	2001	*Amycolatopsis orientalis*	[19]
US6372459B1#	N-acetyl amino acid racemase[2]	2002	*Amycolatopsis orientalis*	[20]
US20070148742A1	Process for the production of amino acids[2]	2007	*S. atratus*, *Amycolatopsis* sp. and *orientalis*	[21]
EP1130108A1&	Methods for racemizing N-acylamino acids and producing optically active amino acids[3]	2001	*Se. benihana*	[22]
US6664083B2&	Methods for racemizing N-acylamino acids and producing optically active amino acids[3]	2003	*Se. benihana*	[23]
JP2001314191A&	Method for racemization for n-acylamino acid and method of production for optically active amino acid[3]	2001	*Se. benihana*	[24]
JP2002238581A	N-acylamino acid racemase gene and its usage[3]	2002	*Deinococcus radiodurans*	[25]
JP2007082534A	Heat-stable N-acylamino acid racemase, method for producing the same and application thereof[4]	2007	*Chloroflexus aurantiacus*	[26]
JP2008061642A	Method for producing D-amino acid[4]	2008	*G. stearothermophilus*	[27]
WO2010050516A1@	Method for producing L-amino acid[5]	2010	*G. kaustophilus*, *T. thermophilus*	[28]
EP2351848B1@	Method for producing L-amino acid[5]	2011	*G. kaustophilus*, *T. thermophilus*	[29]
JP5744521B2@	Method of manufacturing a L-amino acid[5]	2015	*G. kaustophilus*, *T. thermophilus*	[30]
JP2015091265A@	Method for producing L-amino acid[5]	2015	*G. kaustophilus*, *T. thermophilus*	[31]
US9464306B2@	Method for producing L-amino acid[5]	2016	*G. kaustophilus*, *T. thermophilus*	[32]
JP2008307006A	Method for producing L-amino acid[6]	2008	*G. stearothermophilus*	[33]
US20110244530A1	L-succinylaminoacylase and process for producing L-amino acid using it[7]	2011	*G. stearothermophilus*	[34]
US8728771B2	L-succinylaminoacylase and process for producing L-amino acid using it[7]	2014	*G. stearothermophilus*, *C. aurantiacus*	[35]
WO2010067613A1	L-succinylaminoacylase and process for producing L-amino acid using same[7]	2010	*G. stearothermophilus*, *C. aurantiacus*	[36]
WO2018088434A1	Method for producing N-succinyl-hydroxy-D-amino acid and/or hydroxy-D-amino acid	2018	*G. stearothermophilus*, *C. aurantiacus*	[37]
WO2009136500A1	L-succinyl aminoacylase and process for producing an L-amino acid using the same[7]	2009	*G. stearothermophilus*	[38]
JP6048850B2	D- succinylase, and method for producing D-amino acids using the same[7]	2015	(a)	[39]
WO2012002450A1	D-succinylase, and method for producing D-amino acid using same[7]	2012	(a)	[40]
US20030059816A1	Methods for identifying racemases[9]	2003	(a)	[41]
US20080003640A1	*Deinococcus* N-acylamino acid racemase and use of preparing L-amino acid[10]	2009	*Deinococcus radiodurans* NCHU1003	[42]
WO2012140507A1	Production of enantiomerically purified amino acids[11]	2012	*Amycolatopsis* sp. TS-1-60 NAAR/ Double mutant	[43]

Table 1. Patents on NSAR applications. Symbols (*,#,&,@) next to the patent number indicates related patents. [1]Takeda, [2]Evonik, [3]Daicel, [4]Dai Ichi Pure Chem/Toyo Boseki, [5]Kaneka, [6]Toyo Boseki, [7]Toyo Boseki/Sekisui Med., [8]Toyo Boseki/Univ. Kyoto, [9]Thermogen, [10]Chung Hsing Univ, [11]Dr Reddy's Lab/Univ. of Edinburgh. (a) Refers to JP-A-2007-82534 and 2008-61642 JP, but we could not obtain the corresponding documents.

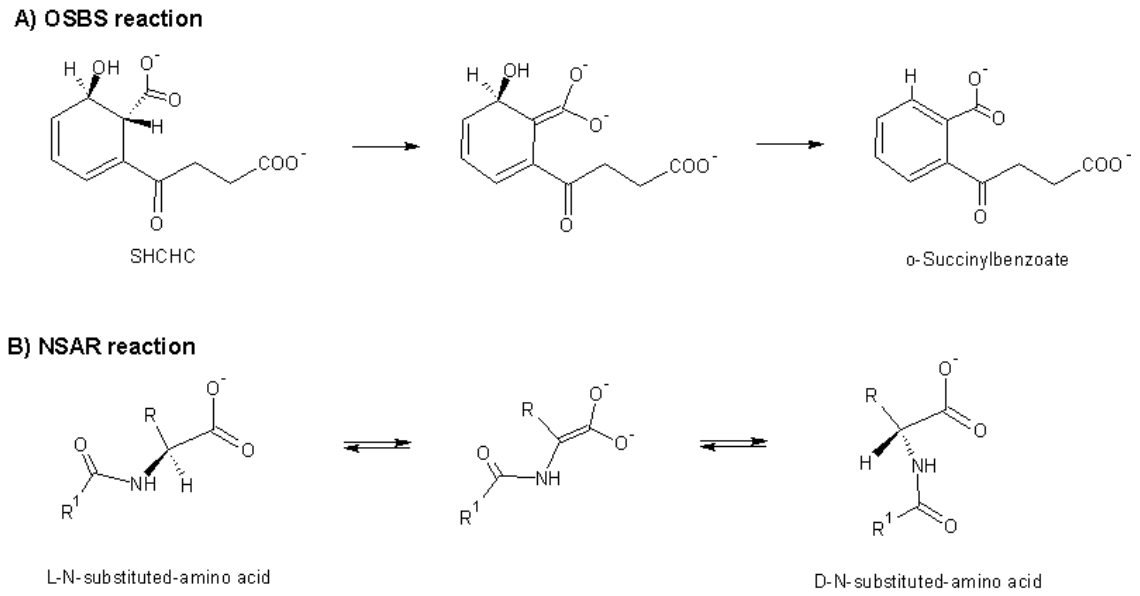


Figure 1. OSBS (A) and NSAR (B) activities catalyzed by different members of the NSAR/OSBS subfamily. N-Succinyl-amino acids (R=succinyl) are likely to be the biologically preferred acyl group [57]. It is important to highlight the similarity between a succinyl-group and the 3-carboxypropionyl group in SHCHC. C) General strategy proposed for abstraction of the α-protons of carboxylic acids by the enolase superfamily.

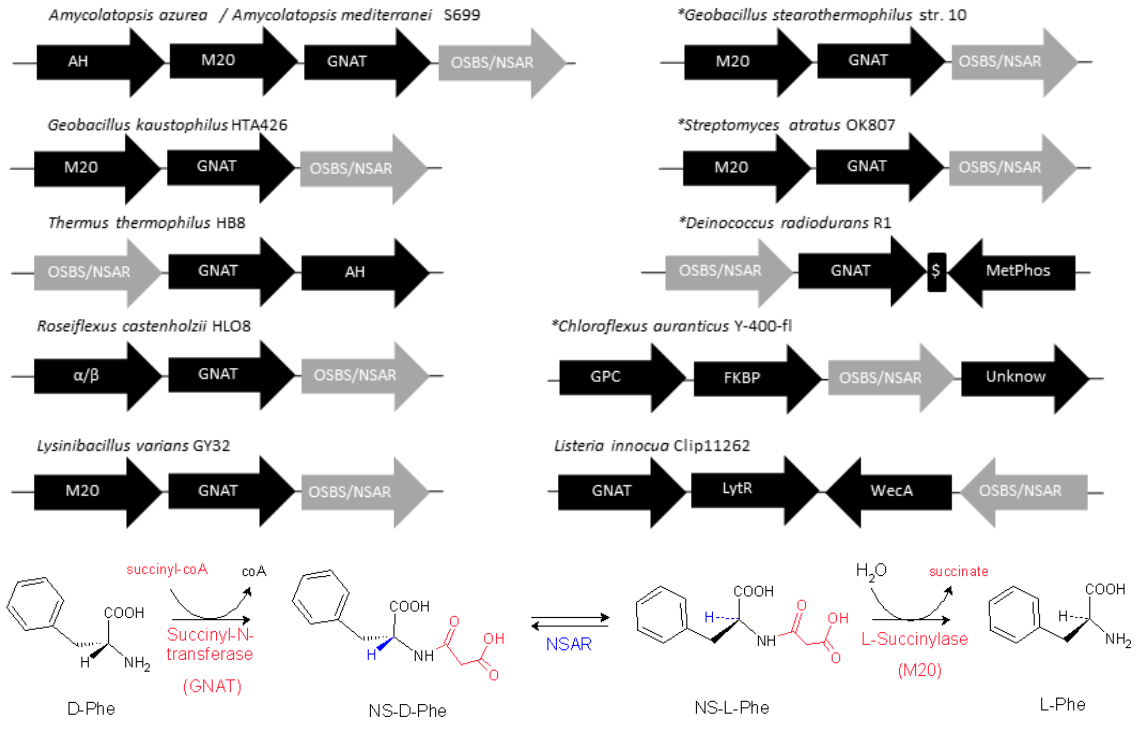


Figure 2. Gene clusters containing NSAR/OSBS members [57,74]. Those clusters from a different strain to the one where NSAR has been reported are marked with an asterisk (*). *Amycolatopsis azurea* (AY271627), *Amycolatopsis mediterrani* S699 (G0FPT7), *Geobacillus kaustophilus* HTA426 (NC_006510.1), *Thermus thermophilus* HB8 (NC_006461.1), *Roseiflexus castenholzii* HLO8 (NC_009767.1), *Lysinibacillus varians* GY32 (NZ_CP006837.1), *Geobacillus stearothermophilus* str. 10 (NZ_CP008934.1), *Streptomyces atratus* OK807 (NZ_FPJO01000025.1), *Deinococcus radiodurans* R1 (AE000513.1), *Chloroflexus auranticus* Y-400-fl (NC_012032.1), *Listeria innocua* Clip11262 (NC_003212.1). Abbreviations are as follows: M20: M20 peptidase superfamily. AH: amidohydrolase superfamily. GNAT: member of the GNAT *N*-acetyltransferase superfamily. α/β: α/β hydrolase superfamily member (probably same function as M20 [74]). MetPhos: metallophosphoesterase. $: tRNA-lysine. GPC: Glutaminyl peptide cyclotransferase; FKBP: FKBP-type peptidyl-prolyl cis-trans isomerase; LytR: Sensory transduction protein LytR; WecA: UDP-*N*-acetylglucosamine-undecaprenyl-phosphate *N*-acetylglucosaminephosphotransferase. Lower panel schematically shows the irreversible pathway for the conversion of D- to L-amino acids discovered in *G. kaustophilus* HTA426 [57]. Phenylalanine is given as an example, but the reader is pointed to [57] where a comprehensive study on the different substrates for the different enzymes in this route was conducted.

After deciphering the natural function of NSAR, huge efforts have been paid on finding the molecular determinants allowing the evolution of the OSBS family and to understand the substrate promiscuity of NSAR/OSBS enzymes [69-74]. Protein Engineering of NSAR [75,76] and the development/improvement of the biotechnological applications of NSARs have also been pursued [63,66,67]. Studies focused on the Pro18-Gly24 loop (known as 20s loop, see below) of AmyNSAR, which flaps upon substrate binding, confirmed its essential character for achieving biologically relevant NSAR and OSBS activity levels [71]. However, studies on the counterpart loop in different members of the enolase superfamily suggest a complex picture to fully understand the molecular determinants guiding enzyme activity in the superfamily. Its divergence contributes not only to the specificity of the activities of the superfamily, but also suggests different roles in the context of different enzymes of the family [70,71]. The origin of the promiscuous NSAR activity was placed near the base of the NSAR/OSBS subfamily tree [72], and thus, it is advisable to look for new NSAR enzymes in this subgroup. Different OSBS/NSAR subfamily members with NSAR activity have been identified, such as those from *Exiguobacterium* sp. AT1b OSBS (ExiOSBS) [72], *Enterococcus faecalis* V583 (EfaNSAR), *Listeria innocua* (LinNSAR) [73], *Lysinibacillus varians* GY32 (LvaNSAR), *Roseiflexus castenholzii* HLO8 (RcaNSAR) and *Amycolatopsis mediterranei* S699 (AmedNSAR) [74]. On the other hand, the OSBS/NSAR subfamily also contains OSBS enzymes with no apparent activity with NAAs, such as *Bacillus subtilis* YtfD[2] or *Staphylococcus aureus* [51,73,74]. The same occurs for the recently described OSBS from *Alicyclobacillus acidocaldarius* (AacOSBS), for which a modest NSAR activity was engineered by a single mutation [74]. In addition, it has been suggested that several species into the Firmicutes subfamily have obtained the NSAR/OSBS gene by lateral gene transfer [72]. The difficulties to find new NSARs would explain why in more than 30 years from their discovery, mainly the NSARs from *Amycolatopsis* and *Bacillus*/*Geobacillus* have been profoundly studied and exploited (Table 1, [10-21,27-38,43,47-53,55,64-68]). *Deinococcus, Streptomyces, Thermus* and *Chloroflexus* enzymes have also found discrete applications in the biotechnological field (Table 1, [7,8,11,12,16,21,25,26,28-

[2] This enzyme shares more than 40 % of sequence identity with NSARs of proved activity, although it has been suggested -but not experimentally demonstrated - that it might have activity with other NxAs, since it only was checked with an NAA substrate [72].

32,35-37,42]). While *Exigobacterium* [72], *Lysinibacillus*, *Roseiflexus*, *Enterococcus* or *Listeria* OSBS/NSAR enzymes have been reported to exhibit NSAR activity [73,74], we could not find any information on whether these enzymes recognize other NxAs; the substrate promiscuity of these enzymes needs to be further studied. Finally, the genome sequences of some microorganisms for which NAAR activity has been detected are available in public databases, and these enzymes might be also worth to study [6,9,44,45,56].

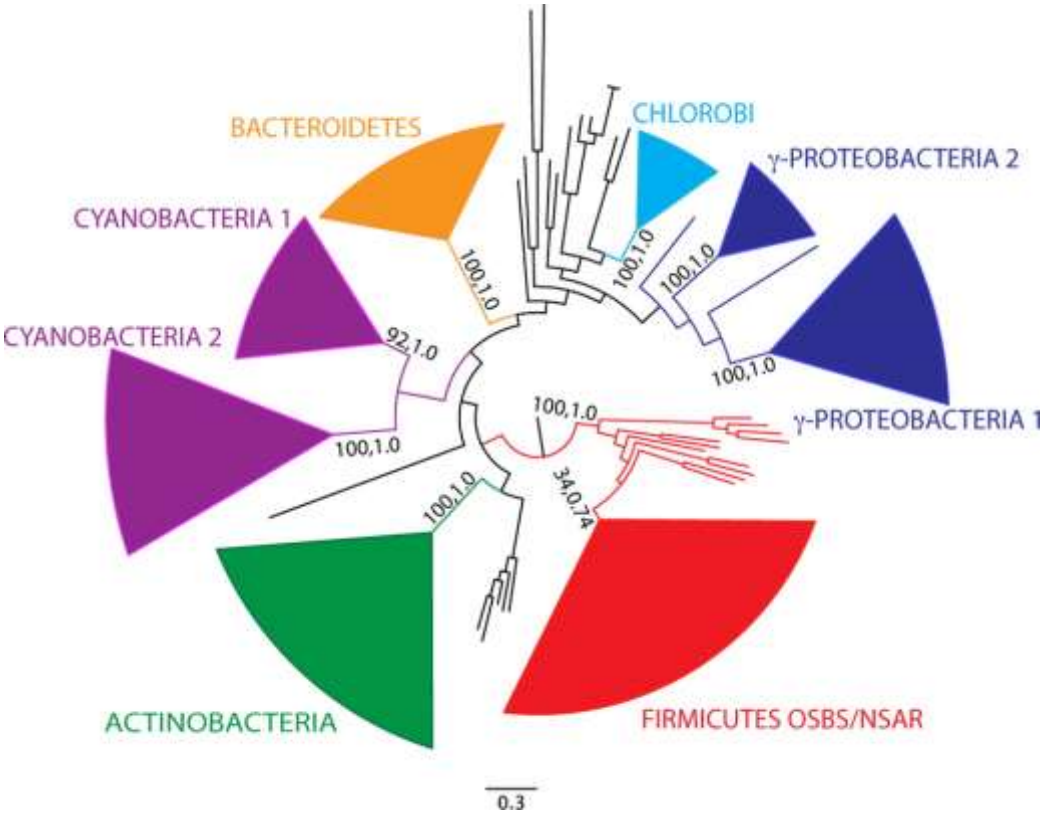


Figure 3. Phylogenetic tree of the OSBS family, showing the division into eight major subfamilies, one of them being the OSBS/NSAR subfamily where the NSAR activity seems to be confined. The reader is referred to [58,69,71,72,74] for deeper insights into the phylogeny of these enzymes "Reprinted with permission from [69]. Copyright 2012. American Chemical Society".

At this point, the reader interested in NSARs with biotechnological potential should understand the difficulties of sequence-based identification of new NSARs. As already highlighted by the numerous works by Gerlt and Glasner, besides the different nomenclature used for these enzymes in the literature, two specific difficulties persist to find new NSARs. Firstly, since different members of the NSAR/OSBS subfamily where

NSAR activity is found do not present this promiscuous activity, and they do not constitute a separate clade, it is complicated to predict NSAR activity from sequence similarity alone (Figure 3) [58,69,70,72]. As previously mentioned, no activity towards NxA has been found within members of this subfamily (*Bacillus subtilis* or *Staphylococcus aureus* [51,73,74]; ExiOSBS shows inefficient NSAR activity [72,74] and AacOSBS does not present activity towards NS-PheGly [74]). Secondly, several works have proved the difficulties to find the determinant allowing the evolution of OSBS family towards NSAR activity [70,71,74]. NSAR activity thus appears to be a recent evolutionary event towards new metabolic activities such as the interconversion of D- to L-amino acids for *Geobacillus kaustophilus*, which to date seems to be confined principally to enzymes in the Firmicutes NSAR/OSBS subfamily [57,58,74]. Odokonyero et al. also highlights that in some cases OSBS activity is a promiscuous, nonbiological side reaction, while NSAR (or another activity, which is yet to be determined) is the primary biological function [74]. The later hypothesis is supported by gene clusters such as those found in *Chloroflexus auranticus* or *Listeria innocua* (Figure 2). With these difficulties in mind, we might argue that new NSAR could be found in a gene cluster similar to that found in *G. kaustophilus*, different to the classical menaquinone biosynthesis operon. Clusters where OSBS/NSAR enzymes appear near a GNAT and/or a M20 superfamily member seem the more reasonable locus to look for new enzymes with biotechnological applications (Figure 2, see also [51,58,74]).

Enzymatic properties of NSARs

NSARs have been successfully purified from wild-type organisms [46,47] or overexpressed/purified from recombinant organisms [48-55,57-75]. Members from the NSAR/OSBS subfamily have been described as dimers (*Enterococcus*, *Listeria*), tetramers (*Geobacillus*), hexamers (*Streptomyces*) or octamers[3] (*Amycolatopsis*, *Deinococcus*, *Thermus*, *Sebekia*) (Table 2). The different quaternary structures of proteins in the enolase superfamily have been suggested to be associated to the evolutionary rate of this superfamily; whereas monomeric members of the superfamily evolved rapidly, the NSAR/OSBS subfamily has been suggested to evolve at a slower rate than other monomeric OSBS subfamilies, since retention of higher-order quaternary

[3] The Molecular masses reported in the corresponding literature for *Sebekia* and *Thermus* do not correspond to octamers, but to higher oligomeric species.

278 structure caused them to retain structural characteristics similar to many of the families
279 in the enolase superfamily [73]. The optimum pH of the enzymes characterized at some
280 detail is found centered in the range 7.5-8.0 (Table 2); optimum temperatures range
281 from 30 °C (*Amycolatopsis orientalis*) to 60-70 °C (*Geobacillus* and *Deinococcus*
282 genera).

Enzyme	Mw (kDa)	Best cation	Opt. pH	Opt. T (ºC)	Inhibition	Ref.
Amycolatopsis sp. TS-1-60	300 octamer	Co^{2+}	7.5	50	EDTA, PCMB, metals (Al^{3+}, Ba^{2+}, K^{+}, Sn^{2+}, Pb^{2+}, Ca^{2+}, Cu^{2+})	[47,78]
Amycolatopsis orientalis	300 octamer	Co^{2+}	8.0	30	EDTA, PHMB, no metal inhibition detected	[52]
Amycolatopsis azurea	321 octamer	Co^{2+}	7.5	40	SH, DTT, β-ME, metals (Li^{+}, Na^{+}, K^{+}, Hg^{2+}, Pb^{2+} Ca^{2+}, Cu^{2+})	[53]
Chloroflexus aurantiacus	ND	Co^{2+}	6.5-8.0	37-70	metals (Cu^{2+}, Ba^{2+}, Zn^{2+})	[26]
Deinococcus radiodurans ATCC 13939*	300 octamer	Co^{2+}	8.0	60	SH	[54]
Deinococcus radiodurans BCRC12827	300 octamer	Co2+	8.0	65	EDTA, dipicolinic acid, metals (Ca^{2+}, Cu^{2+})	[61]
Geobacillus kaustophilus HTA426	ND	Mn^{2+}	7.9	N.D (25)	ND	[57]
Geobacillus kaustophilus CECT 4264	170-177 tetramer	Co^{2+}	8.0	55	HQSA, DTT, β-ME, metals (Hg^{2+}, Pb^{2+} Cu^{2+})	[64,65]
Geobacillus thermophilus CECT 49	174 tetramer	Co^{2+}	8.0-8.5	55-65	No metal inhibition detected	[68]
Sebekia benihana	340 octamer	Co^{2+}	8.0	50	EDTA, PCMB, IAc. No metal inhibition detected	[22]
Streptomyces atratus Y53	244 Hexamer	Co^{2+}	8.0	40	EDTA, PCMB, MalNEt, metals (Al^{3+}, Ba^{2+}, Cr^{3+}, Sn^{2+}, Pb^{2+}, Ca^{2+}, Cu^{2+}, Sr^{2+}; Co^{2+} at high conc.).	[46]
Thermus thermophilus HB8	656 octamer	ND (Co^{2+})	6-9	ND (30-45)	ND	[28,60]

Table 2. Biochemical properties of NSARs from different sources. ND: not determined. EDTA: ethylenediaminetetraacetic acid; PCMB; p-Chloromercuribenzoate; PHMB: p-Hydroxymercuribenzoate. HQSA: 8-hydroxyquinoline 5-sulfonic acid; SH: Salicyl hydroxamate; β-ME: β-mercaptoethanol; DTT: dithiothreitol; MalNEt: *N*-Ethylmaleimide. IAc: iodoacetate. In those cases in which the optimal conditions were not calculated, the numbers in parentheses represent the condition used to asses enzyme activity.* 100% sequence identity with *Deinococcus radiodurans* NCHU1003 NSAR, characterized in [42].

The metalloenzyme character of NSAR is clear since its activity is altered in the presence of metal ions and inhibited by chelating reagents (Table 2). Co^{2+} has shown to be the best cofactor for a vast majority of NSARs (Table 2). However, since metal inhibition at high metal concentrations has been observed, cofactor concentration-dependence needs to be assayed to optimize enzyme activity. As way of example, Co^{2+} showed the best performance at 1 mM with SatNSAR, whereas the activity dropped when using 10 mM of this cation. On the other hand, no inhibition was observed when using up to 10 mM $MnCl_2$, but the activity was slightly lower than with 1 mM Co^{2+} [46]. Other OSBS/NSARs have been scarcely described, such as AmedNSAR, EfaNSAR, ExiOSBS, LinNSAR, LvaNSAR, or RcaNSAR [72-74]; these enzymes were characterized using Mn^{2+} or Mg^{2+} as cofactors, at pH 8.0 and 25º C, but the optimal conditions were not characterized. Similarly, the enzyme from *G. stearothermphilus* NCA1503 was used with Co^{2+}, at pH 7.5 and 37 ºC [79,80]. The effect of sulfhydryl reagents has also been well documented [46,47,52,53,64]. However, no Cys residue has been identified to be involved in the activity of the enzyme, nor do disulfide bridges seem to be necessary for protein folding [54,55,60]. On the other hand, we want to highlight the presence of a totally conserved cysteine residue preceding the metal-binding residue D239 (Cys237 in AmyNSAR; Figure 4), but we cannot ascertain whether this residue is responsible for the effect of sulfhydryl reagents. A deeper characterization of *Geobacillus* NSARs has been conducted [57,64,65,68]. Co^{2+} addition produced an enzyme stabilization of approximately 10-15 ºC [65,68]. Interestingly, the apparent thermal denaturation midpoint (Tm_{app}) for *G. stearothermophilus* CECT 49 NSAR (GstNSAR) was 77.0 ºC, higher than the thermal midpoint calculated from the residual activity after preincubation, showing gradual loss of activity at preincubation temperatures over 50-60 ºC [65,68]. These differences could

be explained by the equilibrium model: enzymatic activity might be lowered or lost below the apparent unfolding temperature as a result of temperature-induced conformational changes at the active site from an optimum configuration for substrate binding to a less optimum one [81,82]. This conjecture is in agreement with the expected flexibility of a promiscuous active site, and is further supported by quantum mechanics/molecular mechanics (QM/MM) analysis in the case of NSAR [83].

NSAR	Substrates recognized	Not recognized	Ref.
Streptomyces atratus Y-53	**NAAs (D- & L-)**: Met, Ala, Leu, Phe, Trp, Val; N**FAAs (D- & L-)**: Met; **NChAAs (D- & L-)**: Phe, Val	**AAs (D- & L-)**: Met, Ala, Leu, Phe, Trp, Val	[46]
Amycolatopsis. sp. TS-1-60	**NAAs (D- & L-)**: Met, Ala, Leu, Phe, Trp, Val; NA-D-Phegly, NA-D-Napala, NA-L-Tyr; **NChAAs (D- & L-)**: Phe, Val; NCh-L-Leu; **NFAA (D- & L-)**: Met; **NPrA (D- & L-)**: Met; **NBtA (D- & L-)**: Met; **NSAs (D- & L-)**: Met, PheGly; NBz-D-Ala; NC-D-Met; L-Ala-L-Met	**AAs (D- & L-)**: Met, Val; NM-D-Phe, NM-L-Phe, N-M-L-Leu; NA-D-Met (methyl ester), NA-L-Met (methyl ester)	[47, 78]
Amycolatopsis orientalis subsp. lurida	**NAAs (D- & L-)**: Met, Phe, Val; **NAAs (D-)**: Ala, ABA; NA-D-Tyr; NCh-L-Phe	NA-L-tert-Leu, NA-D-Napala, NBz-LPhe	[52]
Amycolatopsis azurea	**NAAs (D- & L-)**: Met, Tyr, Val, Leu, Phe, Ala; **NAAs (D- & L-)**: Ile, Thr; **NChAAs (D- & L-)**: Phe	NA-L-Arg, D-Met,L-Met	[53]
Chloroflexus aurantiacus	NS-DL-tertLeu, different NAAs and NFAs,		[26,35]
Geobacillus kaustophilus HTA426	**NSAs (L-)**: Ala, Val, Leu, Ile, Met, Phe, Tyr, Trp, Ser, Cys, Asn, Gln, Asp, Glu, Lys, Arg, His	NS-L-Pro NS-L-Thr (activity not detected)	[57]
Geobacillus. kaustophilus CECT 4264	**NAAs (D- & L-)**: Met, Phe, Trp, Ala, Asn; **NCAs (D- & L-)**: Met, ABA, Norval, NorLeu, HPhe; **NSAs (D- & L-)**: Ala, Phe; **NFAs (D- & L-)**: Met, Ala, Trp, ABA, NorLeu, PheGly, ethionine, HPhe;		[64, 67, 68]
Geobacillus stearothermophilus CECT 49	**NSAs (D- & L-)**: Phe *;* **NAAs (D-)**: Met*;* **NCAs (D-)**: Met, NorLeu; **NFAAs (D- & L-)**: Met, Norleu, ABA, Norval; Best substrate: NF-D-HPhe (among NFAAs)		[68]
Geobacillus stearothermophilus NCA1503	**NSAs (racemic)**: Phe, Trp; **NSAs (L-):** Phe, Val, Ser, Trp; **NAAs (L-)**: Phe		[79, 80]
Deinococcus radiodurans ATCC 13939	**NAAs (D- & L-)**: Met, Phe; **NAAs (L-)**: Gln, Leu, Trp; **NChAAs (L-)**: Phe	**NAAs (L-)**: Arg, Pro, Lys, His; N**AAs (D-)**: Asp, Glu	[54]
Deinococcus radiodurans CECT833	NS-D-Phe, NS-L-Phe; NF-D-Met, NF-D-Norleu; NA-D-Met; NC-D-Met, NC-D-Norleu		[68]
Deinococcus radiodurans BCRC12827	**NAAs (D- & L-)**: HPhe; **NCAs (D- & L-)**: HPhe; **NCAs (L-)**: Gly, Ser, Ala	**NCAs (L-)**: Arg, Trp, His	[61,62]
Thermus thermophilus HB8	*N*-ac-D-Met; **NSAs (racemic)**: 4-Br-Phe, Phe, 3-F-Phe, Napala, 2iGly, 6hGly		[28]
Sebekia benihana IFO 14309	**NAAs (D-)**: Met, Ala, Phe, Asp, Leu, Val, Trp		[22]

Table 3. Substrate promiscuity of different NSARs. NSA: *N*-succynil-amino acids; NAA: *N*-acetyl-amino acids. NFA: *N*-formyl-amino acids. NCA: *N*-carbamoyl-amino acids. NChA: *N*-chloroacetyl-amino acids. NPrA: *N*-propionil-amino acids: NBtA: *N*-

butyril-amino acids. NBz; N-benzoyl-amino acids. NM: N-Methyl-. ABA: 2-aminobutyric acid. Phegly: phenylglycine. Napala: naphtylalanine. tertLeu: Tert-leucine; Norleu: norleucine; Norval: norvaline; HPhe: homophenylalanine. 3-F-ophe, 2iGly: 2-indanyl-Gly. 6hGly: 6-heptenyl-Gly.

Besides the "natural" NSAs racemization, the activity towards different NAAs, NCAs, NChAs, NBtAs, NPrAs, NBzAs or NFAs has been shown (Table 3). NSARs have also been shown to racemize *N*-succinyl-hydroxy-L-amino acids [37] or L-Ala-L-Met [47]. Besides the enzymes presented in Table 3, RcaNSAR, LvaNSAR, AmedNSAR, AacOSBS (Y299I mutant), EfaNSAR, LinNSAR and ExiOSBS are active towards NS-D- or NS-L-PheGly [72-74] (Table 4).

Substrate	**NSAR**	**k_{cat}/K_M order ($M^{-1}\cdot s^{-1}$)**	**Ref**
Several NAAs	AmyNSAR (WT and mutants)	10^2-10^3	[75,76,78]
NA-D-Met	*A. azurea*	10^2	[53]
Several NAAs	GkaNSAR[$]	10^1-10^3	[64]
NA-HPhe	DraNSAR	10^2	[61]
NC-Met	GkaNSAR[$]	10^3	[64]
Several NFAs	GstNSAR	10^2-10^4	[68]
Several NSAs	GkaNSAR	10^3-10^4	[57]
Several NSAs	GkaNSAR[$]	10^4	[64]
NS-Met, NS-PheGly	AmyNSAR	10^4-10^5	[71,72,78]
NS-L-PheGly	DraNSAR	10^5	[73]
NS-L-PheGly	TteNSAR	10^4	[73]
NS-L-PheGly	EfaNSAR	10^5	[73]
NS-L-PheGly	LinNSAR	10^3	[73]
NS-L-PheGly	ExiOSBS	10^1	[72]
NS-D-PheGly	LvaNSAR	~10^3	[74]
NS-D-PheGly	RcaNSAR	10^3	[74]
NS-D-PheGly	AmedNSAR	10^4	[74]

Table 4. Catalytic efficiencies shown by NSAR with different NxAs for which kinetic parameters have been studied at some detail. HPhe: homophenylalanine. PheGly: phenylglycine. [$] strain CECT4264.

Few studies have reported detailed kinetic and affinity parameters of NSARs towards different NxAs (Table 4). The best NxA substrate found to date for an NSAR yields catalytic efficiency values in the range of 10^1-10^5 $M^{-1}\cdot s^{-1}$ (NS-PheGly, Table 4). We previously demonstrated that GstNSAR achieved catalytic efficiencies in the 10^2-10^4 $M^{-1}\cdot s^{-1}$ range with different NFAs, similar to the natural NSA substrates [65,68]. Our results proved that NFAs could be a better substrate than the original NAAs (10^1-10^3 $M^{-1}\cdot s^{-1}$, Table 4), and its use might be advantageous in some cases. Apart from these

biochemical studies, most of the reports on NSAR substrate promiscuity only report the percentage of the activity of NSARs towards different D- and L-NxAs [45-47,50,52,53,54]. From these values, no clear preference of NSARs for one of the enantiomers could be ascertained (e.g., the relative activities for SatNSAR and AmyNSAR were NA-L-Met > NA-D-Met, but NA-D-Val > NA-L-Val [46,52]). No clear conclusion can neither be extracted from the biochemical parameters on different NSARs towards the D- or L-enantiomers [65,68,78]. Binding studies conducted with GstNSAR mutants showed also slight -but not conclusive- preference for D-NFAA binding [68]. Whereas this slight preference towards D-enantiomers would agree with the expected enantioselectivity of an NSAR participating in the irreversible conversion of D-amino acids to their L-enantiomers [57], we believe that the experimental data available does not allow formulating a general conclusion on NSAR enantioselectivity.

Structural features of NSARs

The first X-Ray structure of an NAAR belonged to the *Deinococcus* genus (DraNSAR) [54], although the structure from AmyNSAR was reported almost simultaneously [55]. Before these X-Ray structures were deciphered, different structural aspects of NSARs were experimentally determined. The metal-dependence of NSARs was reported in the mid-90s [46,47]. Classification into the enolase superfamily was proposed by the end of the millennium [51]. Sequence comparisons of different members of the enolase superfamily suggested the role of three carboxylate groups from Asp/Glu residues in the metal-binding site [53,77], which were initially incorrectly assigned for AmyNSAR [48]. Based on the sequence similarity to other members of the superfamily, Lys163 was suggested as a catalytic residue [48] (Figure 4). In a seminal work on the residue conservation of the enolase superfamily, two bases (Lys163 and Lys263) were proposed in the active site of NSAR as responsible for catalysis [77]. The existence of a KXK motif containing the (S)-specific base was highlighted in different members of the enolase superfamily; it appears to be diagnostic of the presence of functional groups that are involved as electrophilic and general basic catalysts, respectively. Mutagenesis studies supported the two-base mechanism for racemization [51,78].

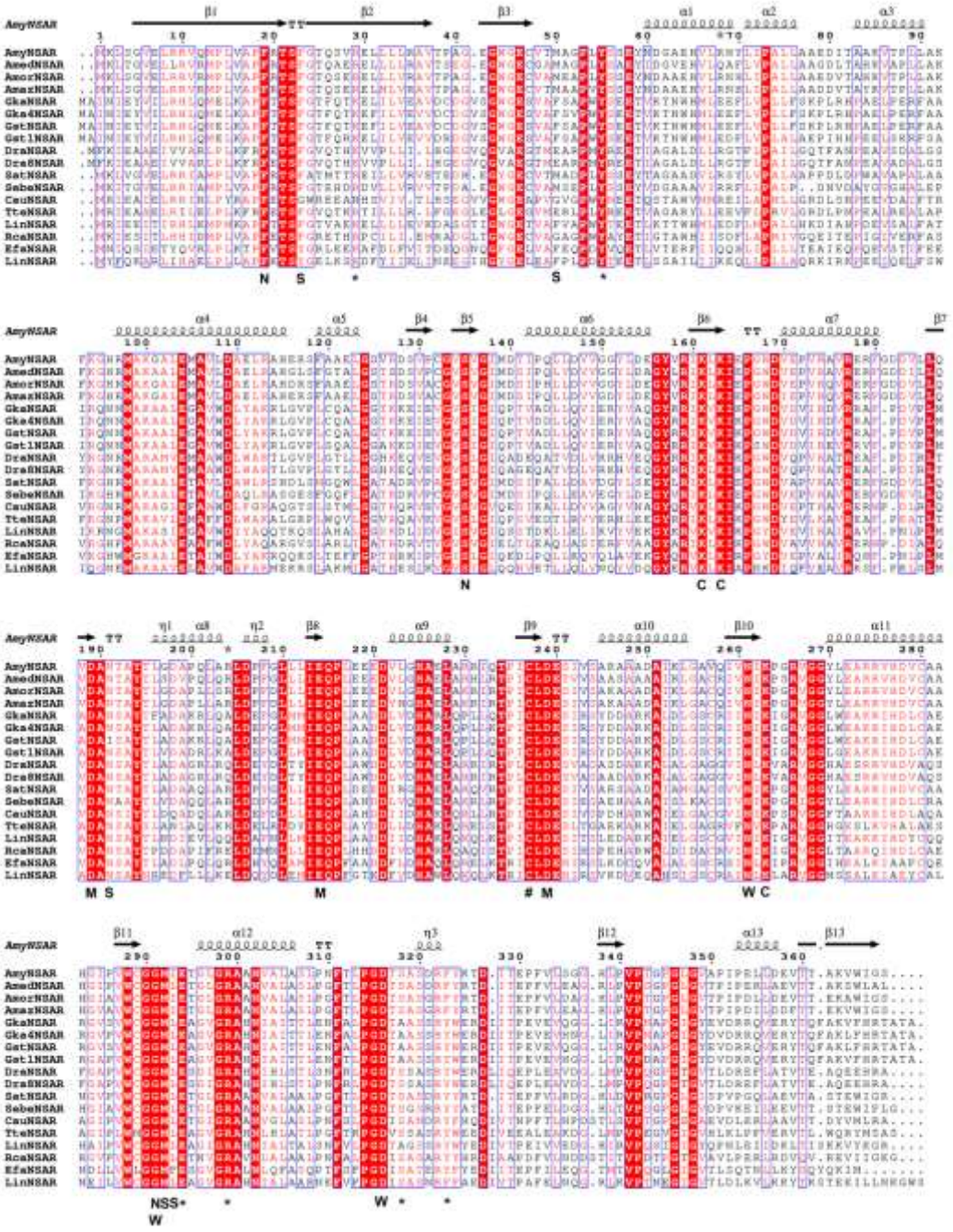


Figure 4. Sequence alignment of OSBS/NSAR enzymes with biotechnological potential generated with the Espript software [101], using BioEdit to generate the alignment used as input, together with the secondary structure of AmyNSAR (PDB ID 1SJA, chain A [55]). GenBank accession numbers available are indicated into bracket following to the corresponding organism. AmyNSAR, *Amycolatopsis* sp. TS-1-60, (BAA06400) [48]; Ammedit, *Amycolatopsis mediterrani* S699, (WP_013225317.1) [51,74]; AmorNSAR, *Amycolatopsis orientalis* subspecies lurida, (CAC00653) [52]; AmazNSAR, *Amycolatopsis azurea*, (AF335269_1) [53]; GkaNSAR, *Geobacillus kaustophilus*

HTA426, (WP_011230427) [51,57]; Gka4NSAR, *Geobacillus kaustophilus* CECT4264, (ABZ81711) [64]; GstNSAR, *Geobacillus stearothermophilus* CECT49, (identical to WP_021321420) [68]; Gst1NSAR, *Geobacillus stearothermophilus* NCA1503, (BAT31602.1) [80]; DraNSAR, *Deinococcus radiodurans* ATCC 13939, (1R0M) [54], Dra8NSAR, *Deinococcus radiodurans* CECT833, (identical to WP_010886692) [68]; SatNSAR, *Streptomyces atratus* Y53, (WP_072488525) [46,51]; SebeNSAR, *Sebekia benihana* IFO 14309, (extracted from [23]); CauNSAR, *Chloroflexus aurantiacus* (WP_012256750) [35]; TteNSAR, *Thermus thermophilus* HB8, (BAD70697.1) [28,60]; LiNSAR, *Listeria innocua* Clip11262 (WP_010991324.1); RcaNSAR, *Roseiflexus castenholzii* HLO8 (WP_012120942.1); EfaNSAR, *Enterococcus faecalis* V583, (WP_002387683.1); LinNSAR, *Lysinibacillus varians* GY32 (WP_025220731.1). The residues at binding distance of NAA in the catalytic pocket of AmyNSAR are marked (PDB IDs 1SJA; C: carboxyl group-binding site; W: water-mediated binding to C; M: metal-binding site; S: side-chain-binding region; N: *N*-acetyl moiety binding residues). Additional residues within binding distance of NSA in PDB ID 1SJC are also marked (*). The highly conserved Cys residue in NSAR is also marked (#).

X-Ray structures of NSAR/OSBS subfamily members are available from *Deinococcus* (DraNSAR, PDB IDs 1ROM, 1XS2, 1XPY) [54], *Amylocaptosis* (AmyNSAR, PDB IDs 1SJA, 1SJB, 1SJC, 1SJD, 4A6G, 5FJO, 5FJP, 5FJR, 5FJU, 5FJT) [55,75 and Sanchez-Carron et al., unpublished results], *Thermus* (PDB ID 2ZC8) [60], *Enterococcus* (PDB ID 1WUE) [73] and *Listeria* (PDB ID 1WUF) [73], confirming the enolase fold. We have also recently solved the structure from GstNSAR at 2.8Å (to be published). The DraNSAR and AmyNSAR structures are those which have been studied in higher detail.

NSAR monomers are composed of a mixed α/β capping domain and a central $(\beta/\alpha)_7\beta$ barrel domain [54] (Figure 5A). Initial inspections of the DraNSAR structure revealed the existence of a disordered loop between two β-strands (residues 24-33 in PDB ID 1R0M, 20s loop), with a proposed role in ligand binding (Figure 5A). Metal-bound structure confirmed the residues involved in metal binding, where a single Mg^{2+} cation is present in each monomer, coordinated by Asp195, Glu220, Asp245 (Figure 4). The Lys170-Asp195-Glu220-Asp245-Lys269 framework was also identified as responsible

for catalyzing a putative 1,1-proton exchange of NAA. The essentiality of a common catalytic platform was reflected by the conservation of this framework in different enolase superfamily members, enabling these enzymes to share the common ability to abstract α-protons of various carboxylic acids efficiently [77].

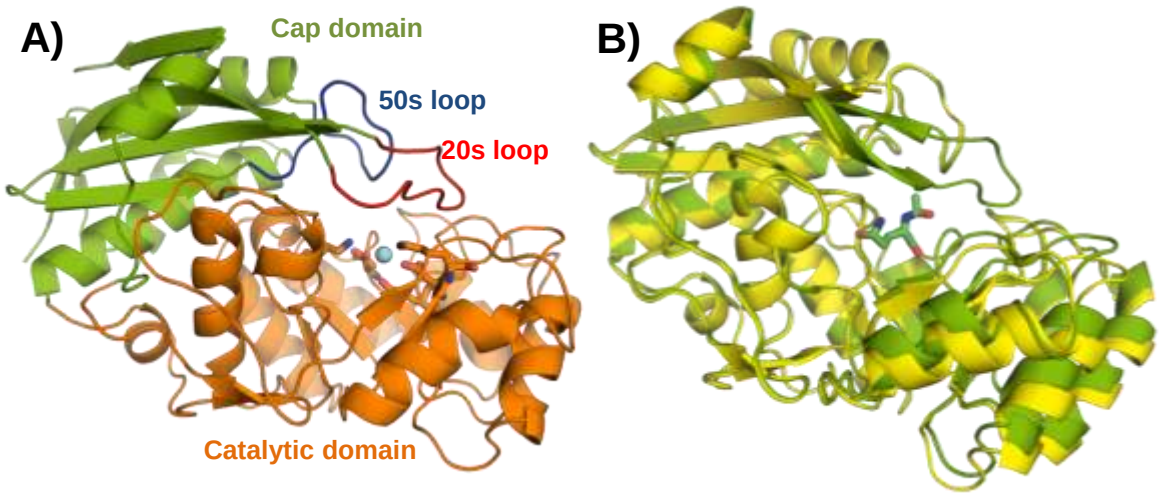


Figure 5. A) Monomeric structure of DraNSAR showing the two domains forming the overall architecture (PDB ID 1XPY; cap domain (green) and catalytic domain (orange). The relative position of the catalytic site, containing the Lys170-Asp195-Glu220-Asp245-Lys269 framework, is shown in stick mode. The catalytic cation is shown as a light blue sphere. The 20s (red) and 50s (blue) loops are also shown. B) Superposition of Mg^{2+}- (PDB ID 1XS2, yellow) and Mg^{2+}/NA-L-Gln (PDB ID 1XPY, green, ligand shown as sticks) bound DraNSAR [54]. Clamp-like movements of the catalytic domain account for ligand-fitting.

DraNSAR undergoes ligand induced-fit movement upon substrate binding (Figure 5B), showing the catalytic domain approaching to the cap domain, with a difference of approximately 15º. In the bound structure, the flexible 20s loop in the cap domain is well defined, demonstrating its key role in enzyme activity. Furthermore, the differences observed in the catalytic domain upon structure superposition (Figure 5B) most likely indicate that it can approach the lid domain thanks to the interaction with the 20s loop. The NAA-bound structures from DraNSAR (PDB ID 1XPY [54]) and AmyNSAR enzymes (PDB ID 1SJA [55]) provide different ligand-binding information: whereas the ligands occupy approximately the same position, the NA-Gln fitted in structure 1XPY appears “rotated” with respect to the ligands fitted in AmyNSAR (specifically, NA-Met

in PDB ID 1SJA, Figure 1SM)[4]. In the NA-Met-bound AmyNSAR structure, the carboxylate moiety of the substrate is at binding distance of Lys161, Lys163, Lys263 and the cation in the metal-binding site. The methionine side chain of the NA-Met substrate is encompassed by groups Phe23, Met 50, Asn191, Met292 and Ile293; the *N*-acetyl-moiety is placed in the environment of Phe19, Ser135 and Gly291 (PDB ID 1SJA) [55] (Figure 6A). Despite no clear interactions of the substrate with these residues, Gly291 and two additional conserved residues (Asn261 and Asp316) bind to water molecules bridging the carboxylate moiety of the substrate (Figure 4, Figure 6A). Besides a displacement of the ligand with respect to NA-Met, the structure bound to NS-Met (PDB ID 1SJC) reveals interactions with the longer succinyl-moiety, some of which also involve water-bridging molecules (e.g. water molecules bound to Glu294, Arg299, or Asp316; Figure 6A). The additional interactions would explain the preference for the natural NSA substrate [55]. Despite the modest resolution of the corresponding structures, superposition of different substrate-bound WT and mutant AmyNSAR structures reveal notable differences in cation and ligand positioning (Figure 6B) with only slight changes in the catalytic center residues. The rearrangement observed in the 20s loop as a result of substrate-induced fit supports its role in substrate binding. Mutation of residues in the 20s loop suggested its importance in enzyme activity and substrate binding [71]. Deletion of residues 18-26 decreased the efficiency of OSBS and NSAR activities by 4500-fold and 25000-fold respectively, although the efficiency of this mutant was still well above the rate of non-catalyzed reactions. The largest effect of a single mutation in the region 18-24 was the F19A mutation, decreasing 200-fold and 120-fold its OSBS and NSAR efficiency, respectively [71]. The authors suggested hydrophobic interactions between Phe19 and the substrate as responsible for the changes observed. Mutation of other two residues which side chains are facing the catalytic center (Thr21 and Phe23) also resulted in lower K_M values and altered k_{cat} values. The three residues are near Lys163, and their mutation could affect the positioning of this catalytic residue, affecting enzyme activity. Phe19 is also in contact with Ser135 and Phe323, which bind to the substrate. Phe23 is near of the tip of the 20s loop, and interacts with Tyr55, Asn191, and the substrate.

[4] Taking into account the availability of other more recent AmyNSAR ligand-bound structures (e.g., PDB IDs 4A6G or 5FJP) and that the electronic density maps corresponding to DraNSAR-bound structure are not available (PDB ID 1XPY), the fitting of AmyNSAR is used in this review to identify the residues involved in ligand binding. In this sense, it is possible that the side chain-binding region might be incorrectly named in [54].

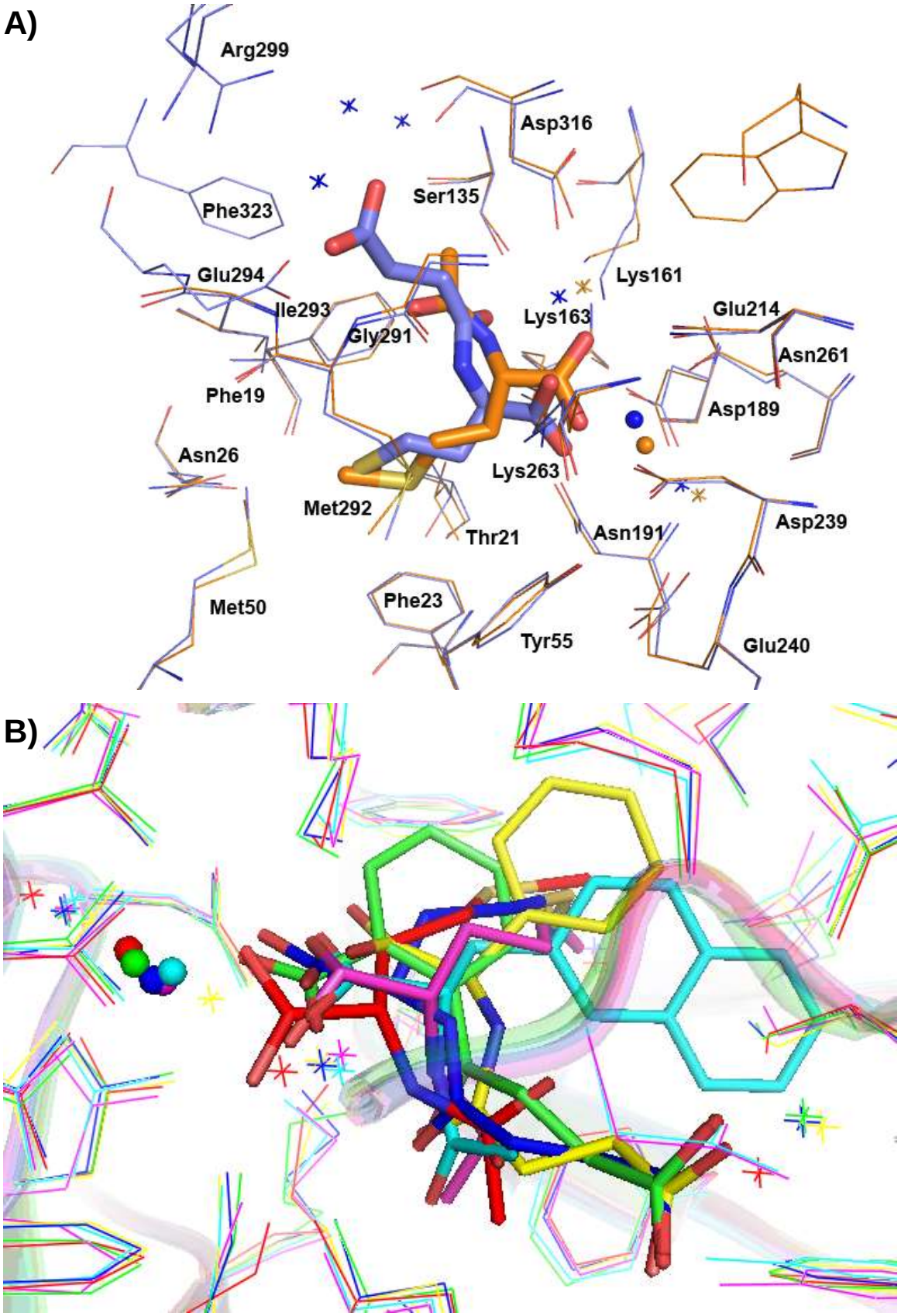


Figure 6. A) Superposition of NA-Met- (PDB ID 1SJA, orange) and NS-Met-bound (PDB ID 1SJC, purple) AmyNSAR structures. Residues in the environment of the

catalytic pocket are shown. Metals are shown as spheres, and water molecules as crosses. B) Superposition of different AmyNSAR-bound structures showing the differences in substrate and metal positioning. NA-Met (PDB ID 1SJA, red), o-succinylbenzoic acid (PDB ID 1SJB, green), NS-Met (PDB ID 1SJC, blue), NS-PheGly (PDB ID 1SJD, yellow), NA-Met (PDB ID 4A6G, G291D/F323Y mutant, violet) and NA-naphtylalanine (PDB ID, G291D/F323Y/I293G mutant, cyan). Metals are shown as spheres, and water molecules as crosses.

Mutation of two surface-exposed residues (Arg20 and Ser22) thought to assist in 20s loop closure through interaction with the cap domain, also resulted in decreased K_M values, although their k_{cat} were only slightly affected [71]. Finally, QM/MM analysis also supports the importance of this loop in AmyNSAR by providing similar interactions to different substrates, which can accommodate in a slight different orientation into the active site (Figure 6) [83].

The NSAR reaction involves a two-base 1,1-proton transfer mechanism, which differs from the one-base syn dehydration of the OSBS reaction[5] [78]. Racemization of NxAs occurs via the base-catalyzed abstraction of the R-proton of the substrate by Lys163 to form a cation-stabilized enediolate anion intermediate; this intermediate is diverted to product with the assistance of an active site acid, which inserts the S-proton (Lys263) (Figure 7) [54,78]. In the opposite direction of the reaction, Lys263 would behave as the base, and Lys163 as the acid. QM/MM studies support this mechanism [83]. Whereas the catalytic framework is highly conserved in the whole enolase superfamily [77], comparison of structural elements related to the activity of the different members of this superfamily, including the 20s and 50s loops, revealed a lower conservation. Despite these regions might support in determining the substrate specificity of different superfamily members, this feature does not appear universal for the whole family [70,71]. The 20s loop of different members of the enolase superfamily is a highly variable element in the evolution of the superfamily; this loop is not present in some members of the superfamily, and it shows no flexibility in other enzymes [70]. However, in the context of the NSAR/OSBS subfamily, the 20s loop has been experimentally shown to play a role in the activity of the enzyme, and possibly to its

[5] A different mechanism is experimentally supported for the OSBS reaction. The reader is referred to [78] for further information.

specificity in two different ways. Firstly, by hydrophobic interactions of Phe19 with both NSAR and OSBS substrates. Secondly, through interactions between surface residues of the 20s loop and residues in the cap domain that seem to set this loop into the closed configuration [71]. These observations reflect the essential character of the catalytic framework in the common strategy in formation of an enolic intermediate in the enolase superfamily (Figure 1), and concomitantly support the divergent evolution of the superfamily from a putative common ancestor into functionally distinct enzymes by variations in structural elements such as the 20s loop [54].

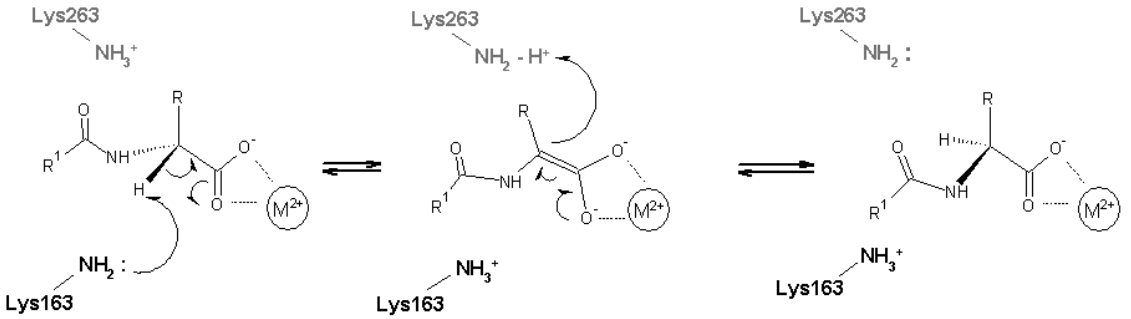


Figure 7. Proposed reaction mechanism for NSAR. Adapted from [54 and 78].

Mutation of specific residues at the region of the enzyme where the *N*-protecting group of the AA binds generated new interactions improving the k_{cat} of the mutated enzyme for different NAAs (2-5 fold), with just slight variations in K_M (mutant G291D/F323Y) [75,76]. This improvement could be explained as a result of new interactions generated by both mutations, mimicking the interactions known to occur between the succinyl-group of the natural substrate and the protein. Another interesting structural aspect determined by Glasner's group is the relevance of the Tyr299 residue in the evolution of OSBS towards NSAR activity [74]. They were able to "evolve" the activity of AacOSBS, lacking NSAR activity, to accept NS-Phegly as substrate by generating a Y299I mutation. They also showed the existence of epistatic constraints, which would complicate even more the structural basis for the determinants governing NSAR promiscuity.

Protein engineering / enzyme immobilization

The plausible link between catalytic promiscuity and evolvability converts the enolase superfamily, in principle, into a good toolbox for protein engineering due to its functional diversity and partially conserved chemical reaction [84,85]. A previous study showed different active-site mutants of *E. coli* OSBS with no detectable activity, but with substantially increased thermal stability [86]. The authors concluded that the results obtained were potentially important with regard to protein design and engineering, since mutants E190A and 3KDED could potentially be used as platforms for protein engineering in (β/α)8-barrels [86]. In the case of NSAR/OSBS, cysteine mutants have been engineered to enhance the enzyme thermal stability by formation of inter- (A68C-D72C and P60C-Y100C) and intra-S-S bonds (E149C-A182C and V265C). Whereas an increase of the T_m could be obtained, the activity was slightly to moderately affected, except for the A68C-D72C variant, which showed similar k_{cat}/K_M ratios to the wild type [87]. Engineering of AmyNSAR was achieved by relying on an *in vivo* selection system that linked the viability of an *E. coli* L-methionine auxotroph to the activity of the improved enzyme, using a simple colorimetric methodology: an evolved G291D/F323Y variant was obtained with up to 6-fold higher activity compared to the wild-type on a range of NAAs [75,76]. The G291D/F323Y variant was coupled with an enantiospecific D-acylase allowing a preparative DKR process for the production of optically pure D-allylglycine [75]. While not intended to specifically improve the catalytic activity of NSAR, a systematic mutagenesis study was conducted on the 20s loop of AmyNSAR and other binding-residues [71]. AmyNSAR mutants P18A, S22R, E165R, D140R/E165R or R20E/D140R/E165R resulted in slightly higher catalytic efficiencies when using the OSBS reaction. On the other hand, only F23A, E165R and R20E/D140R/E165R slightly increased the NSAR catalytic efficiency [71]. Special attention needs to be paid to these positions, since the original work reported expression problems with some of these protein variants [71]. As it has been mentioned previously, AacOSBS has also been engineered to accept NS-Phegly as substrate (k_{cat}/K_M 10^2 $M^{-1}\cdot s^{-1}$) [74].

Some efforts have also been carried out to study the immobilization of NSARs [63,67,88,89]. Enzyme immobilization resulted in a 75% loss of GkaNSAR activity [67] and 90 % with immobilized DraNSAR [63] when compared to the enzymes in solution.

On the other hand, DraNSAR maintained full activity after 40 reaction cycles [63], and over 75 % of the activity remained after ten reactions cycles using GkaNSAR [67]. Despite slight changes/improvements in reaction temperature and thermal stability [63,67], we cannot determine whether immobilization is a cost-effective protocol.

Biotechnological applications of NxAR enzymes

Despite the limited number of NxAR enzymes discovered so far, the racemase activity of these enzymes is widely reported in different DKR processes in the literature. A considerable number of patents have been developed by important industrial companies about the use of NSAR for the production of optically pure amino acids (Table 1). These compounds are of economic interest, since they are used as additives in animal and human foodstuffs, in the pharmaceutical and cosmetics industries, and as chiral synthons in organic synthesis [50,52]. Extensive knowledge on the synthesis of racemic mixtures of amino acids is available (the Strecker reaction represents one of the simplest and most economical methods for the preparation of α-amino acids), and there are also a vast number of methods for the production of many different NxAs [e.g. 57,63,64,66,71]. However, these enzymes have also found application in the production of some oxy-functionalized amino acids, widening their biotechnological application [37,90, Hibi et al, 2017 unpublished results].

From the industrial point of view, it is advisable to bear into mind that *Amycolatopsis orientalis* sub. sp. lurida and AmyNSAR were initially reported to suffer substrate (NAA)/product inhibition [18,51,52]. Despite this drawback, May et al. suggested that the substrate inhibition observed for NSAR from *Amycolatopsis orientalis* sub. sp. lurida might be overcome [50]. Furthermore, substrate inhibition of AmyNSAR was afterwards suggested to be due to the lack of control of the pH [43], and it is reported to be active with 300 mM substrate at pH 8 [75]. In our own experience, GkaNSAR was not inhibited in the presence of substrate concentrations up to 0.5 M [66,67]. Thus, due to the differences found in the literature, inhibition at high concentrations of substrate/product should be assayed with new systems for commercial applications.

Acylase / amidohydrolase process.

Coupling of an NSAR with an L-acylase (E.C. 3.5.1.4) converted the original “acylase process” into a general DKR methodology extensively described for the production of

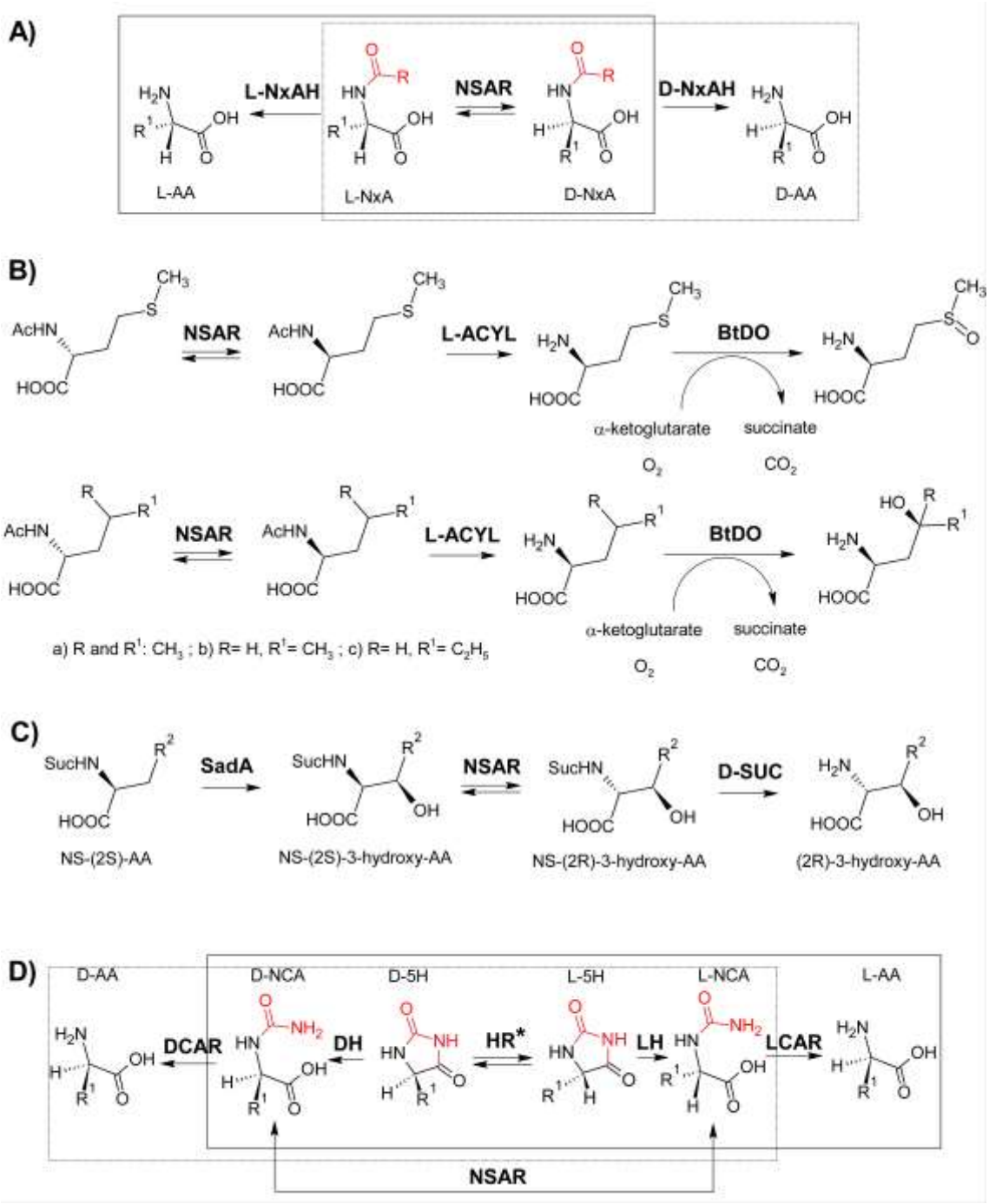
617 optically pure L-amino acids starting from inexpensive D,L-NAA precursors [e.g.
618 15,16,45,49,50,61] (Figure 8A). The tandem NSAR/D-acylase (E.C. 3.5.1.81) has also
619 been experimentally demonstrated (Figure 8A) [21,49,75]. Although NAAs have been
620 the most used substrates with the acylase process, it was early highlighted that other
621 NxAs could also be used as substrates (see Table 3). Since i) several enantioselective or
622 stereospecific amidohydrolases (NxAH) are available under E.C. 3.5.1 group, and ii)
623 some of them have also exhibited a broad substrate spectrum, coupling of an NSAR
624 with one of these enzymes would theoretically allow production of D- or L-amino acids
625 starting from many different NxAs (Figure 8A). The "amidohydrolase process" [66]
626 would be a more general nomenclature to refer to all the different NSAR/NxAH tandem
627 enzyme combinations leading to the production of D- or L-amino acids starting from
628 several NxAs. The NSAR/L-succinylase tandem (Figure 2, lower panel; Figure 8A)
629 described in the D- to L-amino acid conversion pathway [57] has been described in
630 several patents [e.g., 28,29,34,36,38]. In fact, the catalytic efficiencies of L-succinylase
631 for NSAs are in the range 10^3-10^5 $M^{-1}\cdot s^{-1}$, similar to the catalytic efficiency observed for
632 NSARs for the same substrates (Table 4) [57]. Additional enzymatic tandems are the
633 NSAR/L-carbamoylase (E.C. 3.5.1.87) [62,63,66,67,91] or NSAR/D-succinylase
634 [39,40,79,80] (Figure 8A). We previously demonstrated the conversion of NCAAs and
635 NFAAs into the corresponding optically pure L-amino acids, with a productivity of 16
636 mmol L-norleu $L^{-}1\cdot h^{-1}$ (yield >99 %; e.e. 99.5 %), showing no inhibition at high
637 concentrations of substrate or product using immobilized NSAR/L-carbamoylase; 0.5 M
638 of racemic NF-aminobutyric acid could be converted in 85 hours [66,67]. Furthermore,
639 GKaNSAR showed full activity after incubation at 45 ºC for 24 hours [66]. Most
640 noticeably, the use of NF-AAss with an NSAR/L-carbamoylase tandem resulted in an
641 unexpected higher conversion than with the use of NAAs or NCAAs, showing catalytic
642 efficiencies for *Geobacillus stearothermophilus* L-carbamoylase in the range of 10^3-10^5
643 $M^{-1}\cdot s^{-1}$ for NFAAs. These results showed that substrate selection is an important
644 parameter to be considered for the design of a NSAR/tandem enzyme combination,
645 since the efficiencies achieved are comparable to the natural NSA substrates.

646 Although we might have missed experimental evidence in the literature, an additional
647 exploitable enzymatic system is the NSAR/D-carbamoylase (E.C. 3.5.1.77) tandem
648 [91]. As we demonstrated previously, using alternative NxA substrates unexpectedly
649 increased the yield of the NSAR/L-carbamoylase tandem (with NFAAs) [66,67]; since

other NxAs are known to be substrates for NSARs (see Table 3), coupling with stereospecific NxAH recognizing other NxAs could produce alternative amidohydrolase systems with a higher potential to those known to date. Furthermore, the different solubility of substrate/products of the reaction can also be exploited to obtain the product of the reaction *in situ* (e.g., L-homophenylalanine), greatly reducing the cost of operation.

Expanding the use of the amidohydrolase process

Taking advantage of natural metabolic reactions, substrate promiscuity or engineered enzymes, methods can be evolved towards new substrate or products by inclusion of new enzymes. In this sense, enzyme modification of the amino acids produced by any of the possible NSAR/NxAH tandems described above is feasible. Hydroxylation of AAs or NSAs are among the numerous theoretical possibilities [92,93]. 2-Oxoglutarate-dependent oxygenases are a family of enzymes catalysing various different reactions during the biosynthesis of many secondary metabolites, including hydroxylation, dealkylation, desaturation, epoxidation, epimerization, cyclization, and halogenations. Industrially, this class of enzymes has been used mainly for the derivatization of amino acids and in the biosynthesis of antibiotics [94]. A multi-enzymatic cascade expanding the established NSAR/L-acylase process has been proposed for the stereoselective production of γ-oxyfunctionalyzed amino acids [90] (Figure 8B). Coupling of this system with a stereoselective isoleucine dioxygenase from *Bacillus thuringiensis* results in a new approach for the production of L-methionine-(*S*)-sulfoxide and different γ-hydroxy amino acids [90]. Another interesting approach has been presented by Ogawa's group. Taking advantage of the NSAR/D-succinylase system, inclusion of an *N*-succinyl-L-leucine 3-hydroxylase allowed the production of several aliphatic (2R)-3-hydroxyamino acids with high optical activity (Figure 8C). (2R,3R)-3-hydroxyleucine was successfully produced from *N*-succinyl-L-leucine [Hibi et al, 2017 (unpublished results)].



681 Figure 8. Different enzymatic methodologies where NSARs can be applied. A) General
682 scheme on the amidohydrolase process (D-system, dashed line; L-system, full line).
683 NxAH: N-substituted stereospecific amidohydrolase. R=Acyl, NSAR/Acylase tandem
684 (original acylase process); R=carbamoyl, NSAR/carbamoylase tandem; R=formyl,
685 NAAR/carbamoylase tandem. R=succinyl, NSAR/Succinylase tandem. B)
686 Stereoselective production of γ-oxyfunctionalyzed amino acids. L-ACYL, L-
687 aminoacylase from *Geobacillus thermoglucosidasius*. BtDO: stereoselective isoleucine
688 dioxygenase from *Bacillus thuringiensis* [90]. C) Enantioselective synthesis of

enantioenriched (2R)-3-hydroxyamino acids [Hibi et al 2017, unpublished results]. SadA: *N*-succinyl-L-amino acid 3-hydroxylase from *Burkholderia ambifaria* AMMD; D-SUC: *N*-succinyl-D-amino acid desuccinylase from *Cupriavidus* sp. P4-10-C. D) Modified hydantoinase process (D-system, dashed line; L-system, full line). HR*: hydantoin racemase (or chemical racemization when favored); D-CAR: D-carbamoylase; DH, D-activity of hydantoinase; L-CAR: L-carbamoylase; LH, L-activity of hydantoinase [88,91].

Modification of the hydantoinase process

The hydantoinase process is a cheap and environment-friendly method for the potential production of any optically pure amino acid from a wide spectrum of D,L-5-monosubstituted hydantoins. Its main application is the production of optically pure D-amino acids, as most hydantoinases/dihydropyrimidinases exhibit clear D-enantioselectivity [91,95]. Production of L-amino acids with this system is also possible using the "residual" or engineered L-activity of hydantoinases together with an L-specific carbamoylase [95,96]. As demonstrated in the patents by Bommarius et al. (Evonik industries [17]), coupling of an NSAR together with a D-hydantoinase and an L-carbamoylase allows the racemization of the D-NCAA produced by hydantoinase, producing L-NCAA in the reaction medium, which can be then hydrolyzed by the L-carbamoylase to the corresponding L-amino acid. The HYD/NSAR/LCAR system has been further exploited [97] and expanded by inclusion of a fourth enzyme, hydantoin racemase, which speeds up the process by racemization of the remaining L-5-monosubstituted hydantoin for substrates whose chemical racemization is not favored [88,89,98] (Figure 8D).

Conclusions

The increasing demand for non-natural amino acids makes NSAR an interesting candidate for biotechnological applications, despite the initial drawbacks reported on substrate/product inhibition. The use of racemases allows DKR of cheap substrates, an important parameter of industrial interest. Economic aspects of enzyme production, substrate synthesis and/or operational costs can drive the feasibility of different NSAR/tandem enzyme combinations. The substrate promiscuity of NSAR and the other enzymes participating in enzyme cascades together with a NSAR (such as the amidohydrolase process) opens up new opportunities in NSAR-based technology for the

production of optically pure amino acids and enriched hydroxyl-amino acids. The increasing number of applications appearing in the literature reflects the interest on this enzyme, and the newly implemented NSAR/succinylase methodologies predict new strategies in the forthcoming years. Thus, isolation of new NSAR enzymes with different substrate specificities, improved activity, or higher stability continues to be of great biotechnological interest. Since i) NSAR enzymes have been successfully produced recombinantly, and ii) the prices of synthetic genes nowadays is low, a semi high-throughput approach by cloning, expression and activity analysis of different members of the OSBS/NSAR superfamily is a plausible method to find new and efficient NSAR enzymes. If a sequence-based strategy is intended to find new NSAR enzymes with potential biotechnological application, two relevant aspects should be contemplated. Firstly, conservation of residues involved in NSAR activity need to be ascertained before selection [71,74]. Secondly, searching for similar "M20/GNAT/NSAR'' genetic organizations is advisable for new NSAR identification [57,58,68] (Figure 2). Bioinformatics tools for sequence similarity and genome neighborhood networks are freely available to help in this task for neophyte in the *in silico* field [99,100].

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